The Transferrin Receptor at the Blood-Brain Barrier - exploring the possibilities for brain drug delivery

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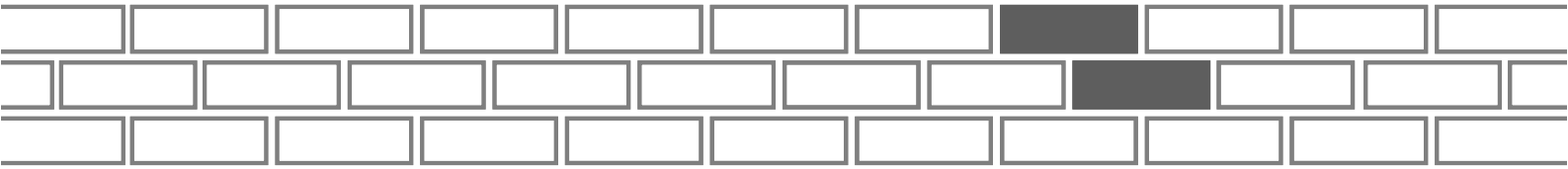
Chapter 2



Drug delivery to the brain:

The transferrin receptor as target

Parts of this chapter will be published in an invited review

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Contents Chapter 2

1. Introduction

The central nervous system is protected by the blood-brain barrier. This barrier limits the transport of exogenous compounds and controls the selective and specific transport of nutrients to the brain. Unfortunately, drugs for the treatment of brain diseases are often not able to cross the blood-brain barrier. Therefore, various drug targeting and delivery strategies are being developed.

This chapter discusses the biology and physiology of the BBB, with a focus on endogenous transport mechanisms, in particularly, the transferrin receptor. Finally, a selection of drug targeting and delivery strategies is reviewed and discussed.

2. The Blood-Brain Barrier

The blood-brain barrier (BBB) is situated at the interface of blood and brain and its primary function is to maintain the homeostasis of the brain. In addition to the BBB, there is a second barrier at the blood - cerebrospinal fluid (CSF) interface, presented by the choroid plexus epithelium (1). Furthermore, the BBB is not uniform throughout the brain, since the capillaries in the circumventricular organs (CVO's) are fenestrated (2, 3). Figure 1 gives a schematic representation of the barriers present in the CNS.

The human BBB has a total blood vessel length of approximately 650 km, and an estimated surface area of approximately 20 m^2, which makes it about 1000 times larger than the blood-CSF or the brain-CSF barrier (4). Therefore, the BBB is considered the most important barrier for solutes to reach the brain (2, 3).

The first evidence for the existence of a barrier between blood and brain was discovered by Ehrlich (1885), who injected the dye trypan blue intravenously and found that, in contrast to other tissues, it did not stain the brain (5). In a second experiment Goldman (1913) injected the dye into the cerebral spinal fluid, after which staining of the brain was observed, but not of the peripheral organs (6). After this discovery, much research has been performed on the biology and physiology of the BBB.

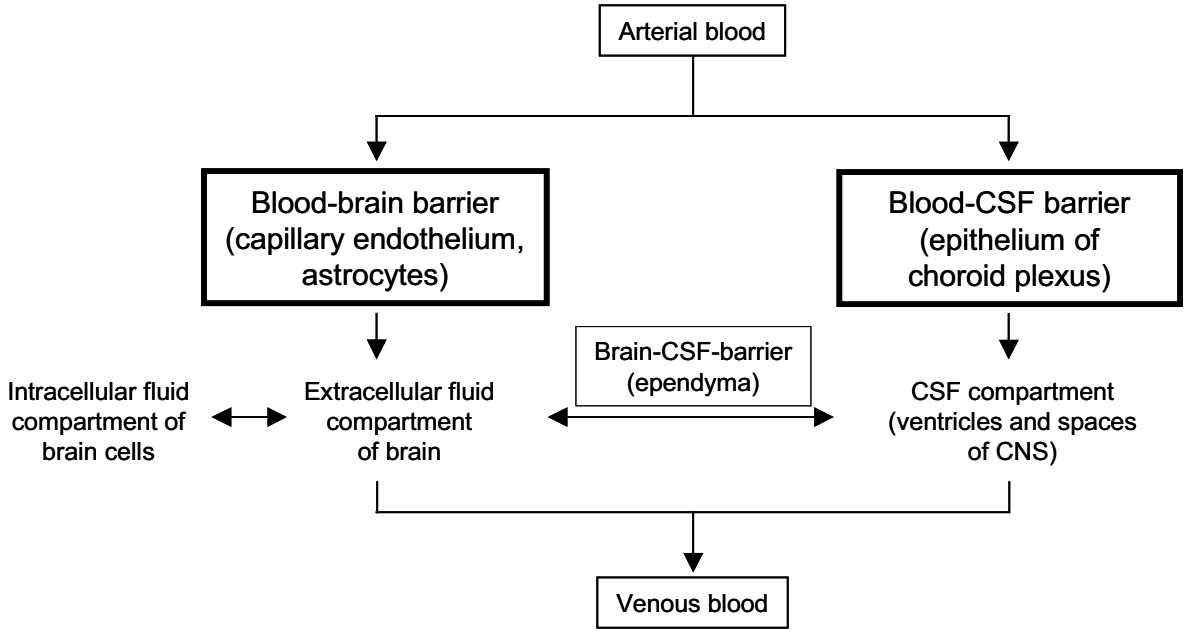

Figure 1: Schematic representation of the blood-brain barrier (BBB), the blood-cerebrospinal fluid (CSF)-barrier and the brain-CSF-barrier. The BBB has the largest surface area, and is therefore considered to be the most important barrier for solutes to reach the brain.

2.1 Blood-brain barrier biology

The BBB is mainly formed by brain capillary endothelial cells (BCEC) (7), although other cells such as, astrocytes, pericytes and neuronal cells also play an important role in the function of the BBB (8). BCEC are different from peripheral endothelial cells, as can be seen schematically in figure 2. BCEC have specific characteristics, such as tight junctions, which prevent paracellular transport of small and large (water soluble) compounds from blood to the brain (7, 9, 10). Furthermore, transcellular transport from blood to brain is limited as a result of low vesicular transport, high metabolic activity and a lack of fenestrae (8). These specific characteristics of the BBB are induced and maintained by the (endfeet of) astrocytes, surrounding the BCEC (7, 11), as well as by neuronal endings, which can directly innervate the BCEC (8, 12). Pericytes also play a role at the BBB, as they share the capillary basement membrane with the BCEC. Their phagocytotic activity forms an additional BBB property (2). Because of these complex interactions between cell types, as well as the dynamic regulation of the BBB properties

(e.g. receptor expression, formation of tight junctions) the BBB is considered to be an organ protecting the brain (13).

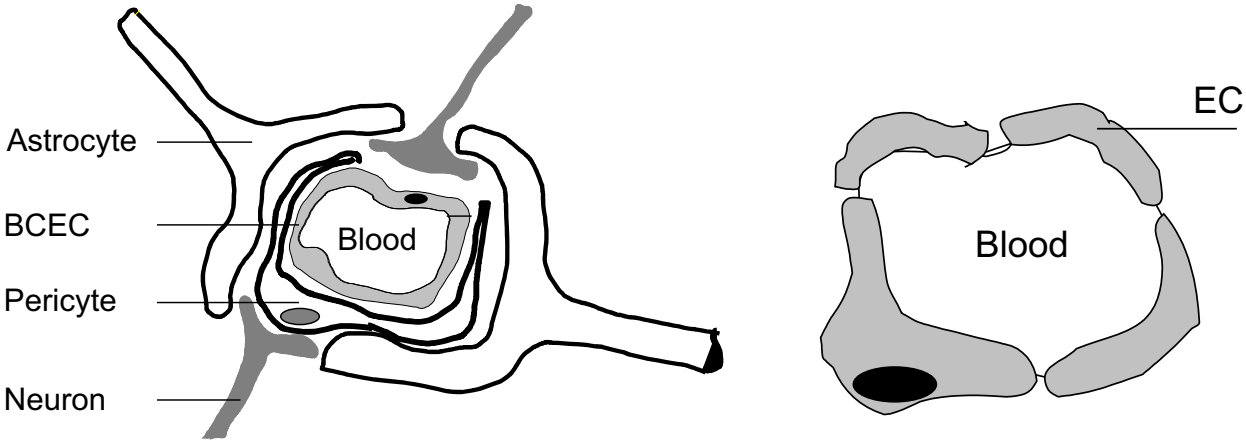

Figure 2: Comparison between a brain capillary endothelial cell (BCEC, left) and a peripheral endothelial cell (EC, right). See text for details. This picture is adapted from Pardridge (8)

2.2. Blood-brain barrier physiology

The function of the BBB is to exclude toxic exogenous compounds from the brain, and to nourish the brain with essential nutrients, such as ions, glucose, amino acids, purines, nucleosides, peptides and proteins (14, 15). Several influx mechanisms exist at the BBB, which can be divided into active or passive BBB transport mechanisms (figure 3). Passive diffusion depends on lipophilicity and molecular weight (16).

Furthermore, the ability of a compound to form hydrogen bonds will limit its diffusion through the BBB (17). In general, Lipinski's rule-of-5, as well as the Abraham's equation can be used to predict the passive transport of a drug molecule across the BBB (18, 19). Transport of hydrophilic compounds via the paracellular route is limited, while lipophilic drugs smaller than 400 – 600 Da can enter the brain via the transcellular route. Active transport systems can be divided into carrier-mediated (CMT), absorptive-mediated (AMT), or receptor-mediated transcytosis (RMT).

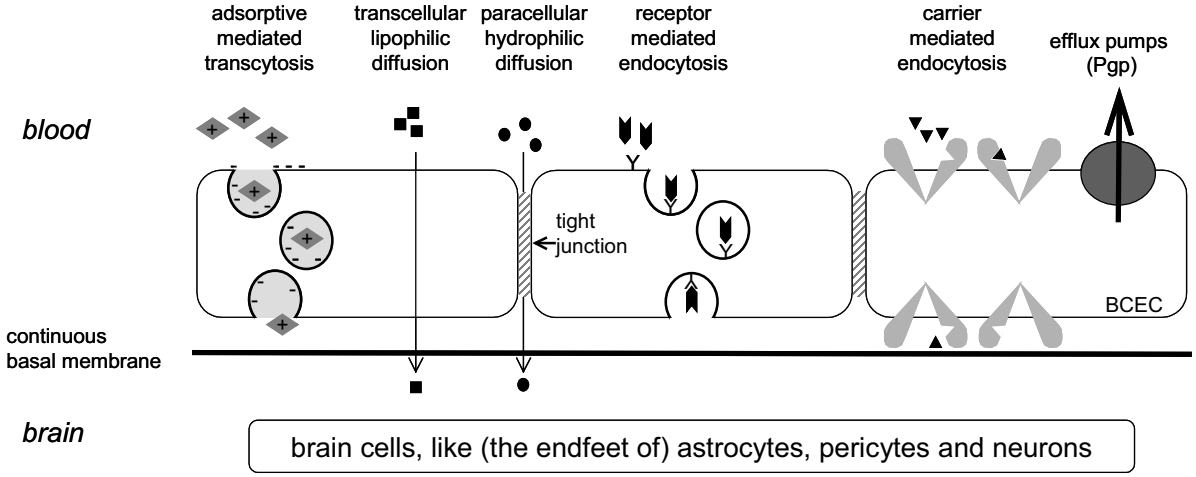

Figure 3: Schematic representation of the transport mechanisms present at the BBB. See text for more details about the transport mechanisms. This picture is adapted from Abbott and Romero (20).

CMT is used for the transcytosis of nutrients, such as glucose, amino acids and purine bases (20, 21). At least eight different nutrient transport systems have been identified, which each transport a group of nutrients of the same structure. Examples are the hexose transporter, which transports glucose and mannose, and the amino acid transporters, which can be roughly subdivided into anionic-, cationic- or neutral amino acid carriers (22). CMT is selective and the transport rate is dependent on the occupation rate of the carrier (21).

AMT is initiated by the binding of polycationic substances to negative charges on the plasma membrane (23, 24). This process does not involve specific plasma membrane receptors. Upon binding of the cationic compound to the plasma membrane, endocytosis occurs, followed by the formation of endosomes.

Peptides and proteins can undergo transport to the brain via RMT. Examples of receptors involved in RMT are the insulin receptor (25), the transferrin receptor (26, 27), and the transporters for low-density lipoprotein (28), leptin (29) and insulin-like growth factors (30). In general, RMT occurs in 3 steps: receptor-mediated endocytosis of the compound at the luminal (blood) side, movement through the endothelial cytoplasm, and exocytosis at the abluminal (brain) side of the brain capillary endothelium (2).

Besides many influx mechanisms, several efflux mechanisms exist at the BBB. The best known is P-glycoprotein (Pgp). Pgp is a transmembrane protein, located at the

apical membrane of the BCEC. It has a high affinity for a wide range of compounds, including cytotoxic anticancer drugs, antibiotics, hormones and HIV protease inhibitors (31). Other multidrug resistance (MDR) efflux mechanisms at the BBB include the MDR related protein (MRP), such as MRP 1, 2, 5 and 6 (32).

In addition, many other transporters are present at the BBB, like the organic anion transporter (influx and efflux), the organic cation transport system (influx) and the nucleoside transporter system (influx) (13, 33).

In conclusion, research over the years has shown that the BBB is a dynamic system, which combines restricted diffusion to the brain for exogenous compounds with specialised transport mechanisms for essential nutrients.

3. The transferrin receptor

The transferrin receptor (TfR) is a transmembrane glycoprotein consisting of two 90 kDa subunits (figure 4). A disulfide bridge links these subunits and each subunit can bind one transferrin (Tf) molecule (27). The TfR is expressed mainly on hepatocytes, erythrocytes, intestinal cells, monocytes, as well as on endothelial cells of the BBB (34, 35). Furthermore, in the brain the TfR is expressed on choroid plexus epithelial cells and neurons (27). The TfR mediates cellular uptake of iron bound to transferrin (Tf).

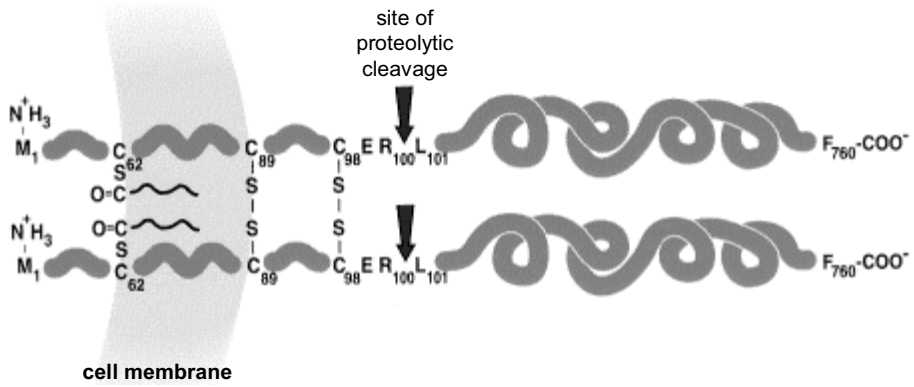

Figure 4: Schematic representation of the transferrin receptor, which is a transmembrane, homo-dimer glycoprotein. Arrows indicate the site of proteolytic cleavage. Standard one-letter abbreviations for amino acids are used (C, cysteine; E, glutamate; L, leucine; M, methionine; F, phenylalanine; R, arginine). Source: http://www.rndsystems.com/asp/g_sitebuilder.asp?bodyId=226

The expression level of the TfR depends on the level of iron supply and rate of cell proliferation. For example, in malignant cells an elevated level of TfR expression is found. This is caused by the high iron requirements for malignant growth (35, 36). The iron concentration determines TfR synthesis and expression via an iron-responsive element (IRE) in the mRNA of the TfR (37, 38). This IRE is also found in the mRNA of ferritin, a protein that can store iron (37). In cases of low iron concentrations, a so-called IRE binding protein stabilises the mRNA of the TfR, which can therefore be translated. The mRNA of ferritin is in low-iron situations less stable and is therefore translated to a lesser extent.

Recently, a second TfR (TfR-2) has been identified (39), which does not contain an IRE in its mRNA. TfR-2 is differentially distributed from TfR and has a 25-fold lower affinity for Tf. Finally, a soluble or serum TfR is present in the circulation (40). During the process of recycling of the TfR, some receptors are shed, in which case they appear in truncated form in the blood circulation (41). It has been shown that serum TfR to ferritin ratios have significant predictive value for differentiating iron deficiency anaemia from non-iron deficiency anaemia (42).

3.1. Transferrin

Tf, the natural occurring ligand for the TfR, is a member of the family of Fe-binding glycoproteins, which also includes lactoferrin, melanotransferrin and ovotransferrin. (34). Plasma Tf is mainly synthesised in the liver, but similar proteins are also synthesised in the brain, testes, and mammary glands. In the brain, Tf mRNA has been found in choroid plexus epithelial cells, oligodendrocytes, astrocytes, and neurons. However, the oligodendrocytes appear to be the major source of brain-derived Tf (27, 34). Furthermore, Tf in the brain is found in neurons and BCEC, although this Tf is probably derived from the extracellular fluid, blood plasma, and brain interstitial fluid by receptor-mediated endocytosis (27).

Tf is a single chain, 80 kDa protein, which is folded into two lobes (27) (figure 5). Each lobe of the Tf molecule can bind one iron ion, a binding that is virtually irreversible at physiological pH (27). Iron is being released as the pH is lowered to values below 6.5. In plasma and other extracellular fluids, Tf is present as a mixture of iron free

(apo-Tf), monoferric Tf, and diferric Tf (holo-Tf). The relative abundance of each form depends on the concentrations of iron and Tf (27, 43).

Tf can also bind other metals, such as aluminium, cadmium, manganese or copper, albeit with lower affinity. It has not been determined yet whether the binding of these metals has physiological significance (27).

Figure 5: A model of human serum Tf, loaded with 2 iron atoms, one in each lobe.
Source: http://srs.dl.ac.uk/arch/DALAI/biology_II.html

3.2. Transferrin receptor – transferrin interaction

Upon binding of Tf to its receptor, the receptor-ligand complex is endocytosed via clathrin-coated vesicles (figure 6, (44)). Subsequently, the endosomes that are formed are acidified to approximately pH 5.5. At low pH Fe^{3+} is released from Tf, and transported to the cytosol via the divalent metal transporter 1 (DMT-1) (44, 45). The remaining apo-Tf has a high affinity for the TfR at low pH and is recycled back to the luminal side of the BCEC. At physiological pH apo-Tf is released from the TfR and is able to acquire iron again. The intracellular Fe^{3+} can be stored in ferritin, or it can be used for mitochondrial activity (44). Furthermore, Fe^{3+} can be exocytosed at the abluminal side,

probably via ferroportin-1, hephaestin and/or hephaestin independent export systems (46).

There is also a second mechanism proposed in which diferric Tf crosses the BBB. Huwyler and Pardridge (1998) have shown that the TfR is present on the abluminal membrane of BCEC (47). Further research by Zhang and Pardridge (2001) has revealed that Tf is rapidly effluxed from the brain and that the efflux of apo-Tf exceeds that of holo-Tf (48). These results indicate that there is a bi-directional transport of Tf (45, 48). However, it has been shown that the iron transport across the BBB exceeds the Tf transport (27). Therefore, the mechanism in which Tf returns to the luminal side after releasing Fe^{3+} intracellularly, is considered the most likely.

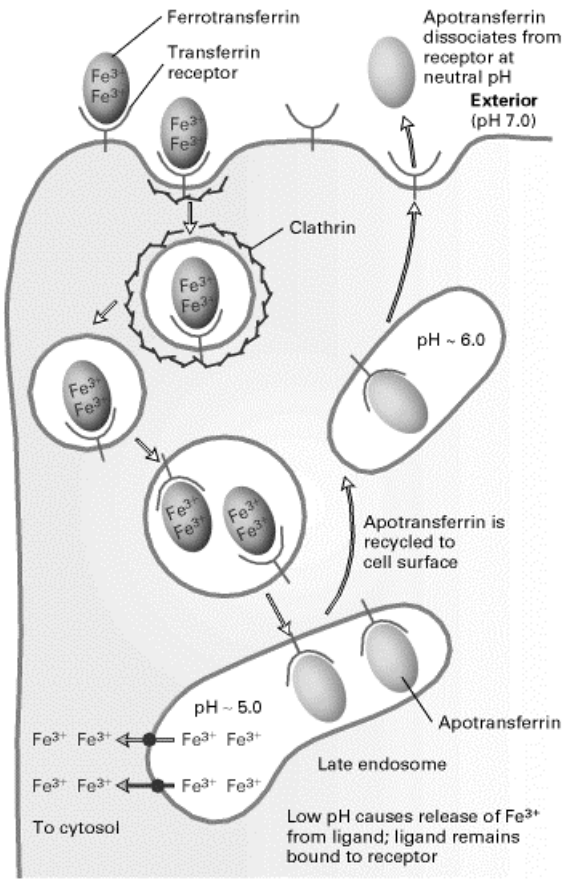

Figure 6: Schematic representation of the TfR internalisation upon binding of Tf to its receptor. See text for details. Source: Molecular Cell biology (section 17.9)

4. Drug targeting and delivery strategies to the brain

For many diseases of the brain, such as Alzheimer's disease, Parkinson's disease, depression, schizophrenia, epilepsy, migraine headache, and HIV infection in the brain no effective drugs are on the market (4). Part of the problem may be the poor BBB penetration of most of the newly developed drugs for treatment of these disorders. This includes approximately 98% of the small molecules and nearly 100% of large molecules, such as recombinant proteins or gene-based medicines (49). Therefore, much effort is put towards targeting and delivery of drugs to the brain. Drug delivery to the brain can be achieved via several methods, including invasive, pharmaco-chemical or physiological strategies.

4.1. Small drug molecules: pharmaco-chemical drug delivery strategies

Pharmaco-chemical strategies, such as making a drug more lipophilic ("lipidisation") or design of a BBB permeable pro-drug can be attractive, but often the pharmacological properties are lost by modification of the drug. Furthermore, "lipidisation" also enhances diffusion through other membranes, thereby increasing side effects. This results in a larger volume of distribution and therefore a lower concentration in blood. Therefore, "lipidisation" results in a minimal change in actual drug delivery to the brain (50).

4.2. Large drug molecules: invasive and disruptive strategies for brain drug delivery

Invasive brain drug delivery strategies, such as direct intracerebral injections of slow release products only allow local delivery (figure 7B). This may be attractive for drug delivery to brain tumours, but not for the administration of drugs against more widespread diseases. Another invasive method is intra-ventricular drug infusion, in which a drug is injected into the CSF of the ventricular organs. However, CSF is completely absorbed from the ventricular organs into the venous circulation (figure 7C).

As a result the infused drug has minimal access to the parenchyma by diffusion (51). In general, invasive strategies are not effective for drug delivery to the whole brain, but only to a localised part of the brain.

Drug delivery through BBB disruption by osmotic imbalance or vaso-active compounds has the disadvantage that the brain can be damaged permanently due to unwanted blood components entering the brain (52).

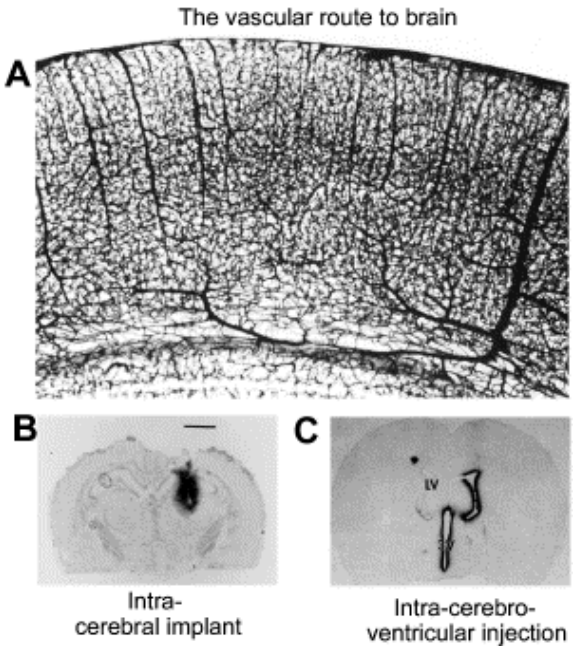

Figure 7: (**A**) Drug delivery via the vascular route enable widespread distribution of the drug to all cells within the brain. (**B**) Minimal diffusion of [^{125}I]-nerve-growth factor (NGF) after intracerebral implantation of a biodegradable polymer. (**C**) ICV injection of [^{125}I]- brain-derived neurotrophic factor (BDNF). The neurotrophin does not distribute into the brain beyond the ipsilateral ependymal surface. From Pardridge (1).

4.3. Physiological drug targeting strategies for brain drug delivery

Physiological drug delivery strategies aim to use endogenous transport mechanisms at the BBB, such as adsorptive-mediated, carrier-mediated or receptor mediated transcytosis. The advantage of the vascular route is the widespread diffusion of the infused drug across the whole brain (1) (figure 7A). This can be explained by the large surface area of the human BBB (approximately 20 m^2). In addition, approximately each neuron has its own brain capillary for oxygen supply as well as the supply of other nutrients. This means that the vascular route is a very promising one for drug targeting and delivery to the brain.

For small drug molecules drug delivery via carrier-mediated transcytosis is possible. Glycopeptide drugs, such as a glycosilated analogue of Met^5-enkephalin are transported

via the hexose transporter GLUT1 (21, 53). In addition, glycosilation of a linear opioid peptide resulted in an improved BBB transcytosis, as well as an improved metabolic stability (54). The best known example of carrier-mediated drug delivery is the transport of L-dopa, a precursor of the neurotransmitter dopamine, in the treatment of Parkinson's disease by the neutral amino acid carrier (16, 55). The disadvantage of carrier-mediated drug delivery is that a drug should mimic an endogenous nutrient (50).

Adsorptive-mediated transcytosis (AMT) is triggered by an electrostatic interaction and can transport larger drug molecules to the brain. The best known compound that is targeted to the brain via this mechanism is cationised albumin (21, 56). An example of a drug transported by AMT is Ebiratide, a synthetic peptide analogue of adrenocorticotropic hormone for the treatment of Alzheimer's disease (57, 58). Ebiratide is positively charged and is resistant to metabolism during the transcytosis across the BBB. AMT is not very specific, but the higher capacity of AMT, compared to receptor-mediated transcytosis, is a favourable property for the delivery of peptides to the brain (4, 21).

A more specific delivery of larger drug molecules or drug carrying particles to the brain can be reached through receptor-mediated transcytosis. Upon receptor-ligand internalisation clathrin-coated vesicles are formed (59). These clathrin-coated vesicles are approximately 120 nm in diameter (60). In this thesis the focus is on the TfR, but also the insulin receptor or the scavenger receptors at the BBB can be used for drug delivery. For the human insulin receptor a monoclonal antibody (HIRMAb) has been developed, which is active in both humans and Old World primates. Approximately 4% of the injected HIRMAb is delivered to the primate brain *in vivo* (61). In addition, the BBB permeability coefficient of the HIRMAb is nine-fold greater than of any other known vector, including vectors directed to the TfR (62). Furthermore, in addition to Tf, P97, or melano-transferrin is also used for drug targeting to the brain. P97 is not taken up via the TfR, but it is selective for drug targeting to the brain, probably via the low-density lipoprotein receptor-related protein (63). However, P97 also activates plasminogen, which could affect angiogenesis as well as blood clotting (64).

Much research has been performed towards a monoclonal antibody against the rat TfR (OX-26) as a targeting vector (49, 65). Furthermore, Fab fragments or small peptides have been developed to target the TfR and to induce receptor internalisation (66). The endogenous ligand, Tf, is also used for drug targeting (67-69). However, the

endogenous concentration of Tf in serum is already around 25 μM, therefore, competition for the TfR might be expected. Tf has been under investigation for the targeting of anti-tumour agents, and for the delivery of gene therapeutics, using polycation-based drug carriers. Furthermore, Tf has been used for the delivery of therapeutically active metals, such as manganese (important as trace element and in several enzymes, such as superoxide dismutase), gallium (as a radiodiagnostic agent) or ruthenium (as a potential anticancer therapy) (70).

4.4. Drug conjugates and liposomes for brain drug delivery

In contrast to pharmaco-chemical, invasive and disruptive strategies, physiological strategies include the application of the drug, a targeting vector and a linker strategy. This linker can either be a direct linker between the drug and the targeting vector, or the drug can be incorporated in a drug carrier, which is tagged with the targeting vector (figure 8). Since the scope of this thesis is on the possibilities for drug targeting to the TfR, this paragraph focuses on the endogenous ligand of the TfR, Tf, and the antibody against the TfR (OX-26) as targeting vectors. A selection of linker strategies will be discussed, focussing mainly on the larger biotechnology drugs, such as proteins and gene medicines.

Proteins can be directly linked to Tf or OX-26 via an avidin-biotin linker, in which a mono-biotinylated protein is conjugated with OX-26 containing a streptavidin or avidin moiety (figure 8A) (71). It is also possible to increase the distance between the drug and the targeting vector, by inserting a PEG-molecule between the biotin moiety and the protein drug (figure 8B). This increased distance reduces the steric hindrance by the OX-26 antibody and increases the possibilities for receptor recognition by the protein drug (72). In addition, proteins or antibodies can be modified at their N-terminus, without loosing receptor-recognition or drug effect. Agents that are used for N-terminus modification are N-succinimidyl S-acetylthioacetate (SATA, (73)), or 2-iminothiolane (Traut's reagent, (74)) to insert a thiol group. This thiol can react with another thiol to form a cleavable disulfide bond. Alternatively, the thiol group can react with a maleimide group to form a stable thio-ester bond.

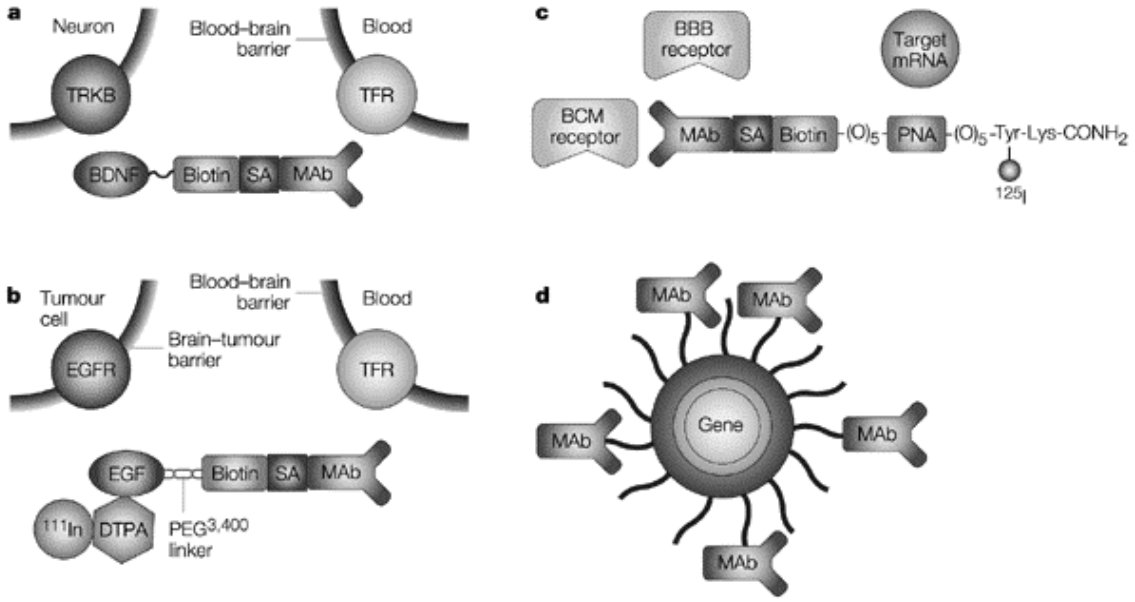

Figure 8: Structures of delivery vehicles for crossing the BBB. (**a**) A direct coupling of the targeting vector (Mab) to the drug (brain-derived neurotrophic factor, BDNF) via the avidin-biotin technology (**b**) Poly(ethyleneglycol) (PEG3,400) is placed between the epidermal growth factor (EGF) and the transport vector to release any steric hindrance of EGF binding to the EGF receptor. (**c**) Structure of an antisense radiopharmaceutical (polynucleic acid, PNA) that is coupled to the Mab to enable transport across both the BBB. (**d**) A double-stranded supercoiled plasmid DNA containing an exogenous gene that is packaged into the interior of an 85-nm liposome. The surface of the liposome is conjugated with ~2,000 strands of PEG, and the tips of 1–2% of the PEG strands are conjugated with a targeting MAb. From Pardridge (87).

Gene medicines, such as antisense mRNA, can also be coupled directly to OX-26 or Tf via the avidin-biotin technology (figure 8C) (75, 76). However, it is necessary to stabilise the mRNA to avoid degradation in serum. Gene medicines can also be incorporated in a drug carrier (figure 8D). This drug carrier can be conventional or sterically stabilised liposomes (77, 78), lipoplexes (79) or cationic amphiphiles (80). Incorporation into a drug carrier protects the drug against degradation in serum. In addition, it is not necessary to modify the drug, thereby preserving the drug properties. For drug targeting to the brain, liposomes are used, consisting of biodegradable phospholipids, which are not immunogenic (81). For prolonged circulation time *in vivo* and increased stability of the liposomes polyethylene glycol (PEG) can be added to the bilayer (82, 83). In general PEG with a molecular weight of 2000 is used at a

concentration of 5%, based on molar ratios. Tf or OX-26 can be coupled to the surface of the liposome, via a maleimide linker, which is attached to a lipid anchor. However, when PEG is attached as well, this "PEG-coat" causes steric hindrance for TfR recognition. Therefore, PEG has been modified with a maleimide group attached to its distal end, enabling Tf or OX-26 coupling (84).

Lipoplexes are formed by positively charged polymers, which can condens negatively charged DNA or mRNA (81, 85). These particles can also be stabilised by PEG. Synthetic amphiphiles are vesicles which are formed by non-biodegrable lipids. SAINT is a well known constituent of synthetic amphiphiles, together with DOPE (80, 86). These drug carriers are often used for transfection purposes, but with PEG their circulation time in the blood can be prolonged as well. Shedable PEG carrying colloidal systems are now under investigation, since the stabilised synthetic PEG-coated amphiphiles showed less transfection. Shedable PEG (PEG-ceramide) is released from the drug carrier in time, after which transfection of the target cells can take place (80, 86). For lipoplexes, as well as synthetic amphiphiles the PEG can be tagged with Tf or OX-26.

5. Summary

In summary, many drug targeting and drug delivery strategies for drug delivery to the brain have been developed. The research described in this thesis will focus on the TfR as a model transport system for drug delivery to the brain. The TfR is highly expressed at the BBB, but also on other cells in the body. Although drug targeting to the brain via the TfR is therefore not selective, it is effective (for review, see (1, 4)). The endogenous ligand, Tf, is used as a targeting vector for drug conjugates as well as liposomes. For *in vivo* applications the use of Tf is limited, since the Tf concentration in serum is high. However, the research described in this thesis uses a mechanistic approach to elucidate the efficacy of endocytosis of Tf-drug conjugates and Tf-tagged liposomes. The results of this research can be used to improve drug delivery to the brain.

6. References

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